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(54) Title: TARGETED SYSTEM FOR REMOVING TUMOR CELLS FROM CELL POPULATIONS

(57) Abstract

A method is presented to remove contaminating tumor cells from a cell population. The method includes labeling individual tumor cells in the population and then killing them with a high energy laser beam. The laser is focused so that it specifically kills the identified tumor cell, but not the remaining cells in the population.

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## Targeted System For Removing Tumor Cells From Cell Populations

### Background of the Invention

#### 5 Field of the Invention

This invention relates to methods and apparatus for specifically isolating tumor cells from a population of non-tumor cells. In particular, this invention relates to methods and apparatus for specifically labeling and thereafter individually killing tumor cells with a focused high-energy beam such as a laser beam.

#### 10 *Description of the Related Art*

Hematopoietic stem cell transplantation is a rapidly growing therapy throughout the world. Hematopoietic stem cells are cells that reside in the bone marrow and lead to the production of all of the body's blood cells. In 1995, over twenty thousand hematopoietic stem cell transplants were performed in the United States. In particular, the treatment of breast cancer with autologous hematopoietic stem cell transplantation has become a widely used cancer therapy.

15 Tumor metastasis is a well-known process by which tumor cells leave their initial location and spread to other parts of the body. Once transported to a new site, the tumor cells begin to grow and populate the new site, thus creating a new tumor. One treatment for patients with metastatic tumors involves harvesting their hematopoietic stem cells and then treating the patient with high doses of radiotherapy or chemotherapy. This treatment is designed to destroy all the patients tumor cells, but has the side effect of also destroying their hematopoietic cells. Thus, once the patient has been treated, the autologous stem cells are returned to their body.

20 However, if the tumor cells have metastasized away from the tumor's primary site, there is a high probability that some tumor cells will contaminate the harvested hematopoietic cell population. In such a case, the harvested hematopoietic stem cells include contaminating tumor cells. It is important to find a mechanism for killing all of the metastasized tumor cells prior to reintroducing the stem cells to the patient. If any living tumorigenic cells are re-introduced into the patient, they can lead to a relapse.

25 The problem of removing tumor cells from hematopoietic cells has been reported during traditional bone marrow harvest procedures (Campana, D. et al. Detection of minimal residual disease in acute leukemia: methodologic advances and clinical significance, *Blood*, 1995 Mar 15, **85(6)**: 1416-34). Similar problems were also found when others attempted to remove tumor cells with the newer method of leukopheresis of mobilized peripheral blood cells (Brugger, W. et al. Mobilization of tumor cells and hematopoietic progenitor cells into peripheral blood of patients with solid tumors *Blood*, **83(3)**: 636-40, 1994).

35 In each of these procedures, the number of contaminating tumor cells ranged from about 10 to 5000 tumor cells per four million mononuclear harvested cells, depending on the chemo-therapeutic drug regimen used for mobilization. Mononuclear cells were obtained by a discontinuous density gradient centrifugation of the entire hematopoietic cell harvest. The total number of mononuclear cells harvested from a patient is normally on the order

of 10 billion cells. Thus, the total tumor burden in a harvest varies from a lower boundary of about 25 thousand cells to a higher boundary of about 12 million cells.

These contaminating tumor cells have been shown by genetic marking to contribute to tumor relapse (Rill, ER et al., Direct Demonstration That Autologous Bone Marrow Transplantation for Solid Tumors Can Return a Multiplicity of Tumorigenic Cells, *Blood*, **84(2)**: 380-383, 1994). Thus, a great need exists for efficient methods for removing all of the tumor cells from a hematopoietic cell transplant (Gulati, SC et al. Rationale for purging in autologous stem cell transplantation. *Journal of Hematotherapy*, **2(4)**:467-71, 1993). A rapid and reliable method for removing all of the contaminating tumor cells would improve the efficacy of hematopoietic stem cell transplantation for a growing number of patients.

Others have attempted to remove contaminating tumor cells from hematopoietic stem cell harvests, but have met with limited success. Several methods of purging the tumor cell populations away from the harvested stem cells have been proposed and tested (A. Gee, Editor Bone Marrow Processing and Purging, Part 5, CRC Press, Boca Raton, Florida, 1991). Thus, the idea underlying all of these purging methods is to separate or destroy the malignant cells while preserving the hematopoietic stem cells that are needed for hematopoietic reconstitution in the transplantation patient.

Some companies and physicians have attempted to purge malignant cells away from populations of non-tumor cells using an immunoaffinity bead-based selection. In this procedure, the total cell population is contacted by immunoaffinity beads. For example, to isolate tumor cells from hematopoietic cells, a first (positive) CD34 selection isolates hematopoietic cells from tumor cells. Binding hematopoietic-cell-specific anti-CD34 antibodies to the immunoaffinity beads allows the physician to specifically remove these cells from populations of non-hematopoietic cells. In some instances, a negative immunoaffinity bead-based selection is also run on tumor or epithelial cell markers by conjugating tumor-specific antibodies to the beads.

Another method that has been tried for removing tumor cells from populations of non-tumor cells involved immunoconjugating a toxic agent to an antibody having specificity for only the tumor cells. In this system, antibodies were bound to chemotoxic agents, toxins, or radionuclides and then contacted with the harvested cell population. Unfortunately, not all of the tumor cells were killed by this treatment.

Other systems for isolating tumor cells from non-tumor cell populations have used the non-specific binding characteristics of hematopoietic cells as the basis for separation. For example, Dooley et al. used these adhesive characteristics to isolate hematopoietic cells with deep bed filtration (Dooley DC et al., A novel inexpensive technique for the removal of breast cancer cells from mobilized peripheral blood stem cell products, *Blood*, **88(10)suppl 1**: 252a, 1996). However, some of the tumor cells were found to isolate with the hematopoietic cells, thus opening the door for a potential relapse by the patient.

In addition, cytotoxic agents, such as 4-Hydroxy-peroxy-cyclo-phosphamide (4HC), have been used to selectively kill tumor cells without damaging the hematopoietic stem cells. Unfortunately, this system also led to lower harvests of hematopoietic cells because the cytotoxic agents weakened or destroyed some of the non-tumor cells.

In other methods, sensitizing agents, such as merocyanine, were mixed with the cell populations which were thereafter photo-irradiated to specifically kill the tumor cells (Lydaki et al. Merocyanine 540 mediated photoirradiation of leukemic cells *Journal of Photochemistry and Photobiology* 32(1-2):27-32., 1996). Also, Gazitt et al. used fluorescence activated cell sorting (FACS) to sort hematopoietic stem cells from tumor cells (Gazitt et al. Purified CD34+ Lin- Thy+ stem cells do not contain clonal myeloma cells *Blood*, 86(1):381-389, 1995). As is known, flow cytometry sorts cells one at a time and physically separates one set of labeled cells from another second set of cells. However, it has been shown that individual neurons can be killed after loading them with the adsorbing dyes used in flow cytometry (Miller, JP and Selverston AI., Rapid Killing of Single Neurons by irradiation of Intracellularly injected dye *Science*, 206:702-704, 1979). Thus, using FACS to separate cell populations is not advantageous because the cell yields can be very low.

In another protocol, Clarke et al. disclosed the use of adenovirus mediated transfer of suicide genes to selectively kill tumor cells (Clarke et al. A recombinant bcl-x s adenovirus selectively induces apoptosis in cancer cells but not in normal bone marrow cells *Proc. Nat. Acad. Sci.* 92(24):11024-8, 1995).

However, most of the methods listed above are based on administering a whole-population based tumor cell separation or killing strategy. Unfortunately, the whole population tumor purging methods listed above do not kill or remove all of the contaminating tumor cells from the harvested stem cell population. In the best case, the residual tumor cell burden remained at 1 to 10 tumor cells for every 100,000 cells present in the initial harvest (Lazarus et al. Does in vitro bone marrow purging improve the outcome after autologous bone marrow transplantation? *Journal of Hematotherapy*, 2(4):457-66, 1993).

Therefore, even using the best available techniques, the number of residual tumor cells that are reintroduced into the patient during autologous stem cell transplantation is on the order of 10 to 2000 cells. Given the rapid exponential growth of tumor cells, such residual tumor cells in the transplant can quickly lead to a patient's relapse.

A still further method utilizing laser technology is described in U.S. Patent Number 4,395,397 to Shapiro. In the Shapiro method, labeled cells are placed in a flow cytometer having a fluorescence detector to identify the labeled tumor cells. A laser beam is used to kill the labeled cells as they pass by the detector. This method suffers from a number of disadvantages. Firstly, once an unwanted cell has passed through the detector/laser region there is no way to check that destruction has been completed successfully. If a tumor cell evades destruction it will inevitably be reintroduced into the patient. Secondly, the focal spot diameter of the laser beam is of necessity greater than the liquid stream cross section. Accordingly, many of the cells in the region of an unwanted cell will be destroyed by the laser beam, including healthy cells.

Another method that utilizes laser technology is described in U.S. Patent Number 5,035,693 to Kratzer. In this method, labeled cell populations are placed on a moving belt. The labeled cells are thereafter identified by a detector and destroyed by a laser. However, this system has many of the same disadvantages as the Shapiro method. For example, because the cells are moving on a belt past the detector in one direction, the method is not reversible. Thus, if a single tumor cell escapes detection, it will be reintroduced into the patient.

Thus, in spite of extensive efforts and many innovative approaches, there exists a great and growing need for methods and systems for eradicating virtually every tumor cell from a harvested cell population. The system and method described herein fulfills this need.

#### Summary Of The Invention

This invention provides a targeted system and method of individually identifying and destroying contaminating tumor cells in a cell population. Using the system of the present invention, virtually every tumor cell can be identified and individually destroyed. Thus, autologous hematopoietic cell transplantation and similar medical techniques can be performed without re-introducing any contaminating tumor cells.

Tumor cells can be identified with the disclosed invention using several approaches. One embodiment includes a non-destructive labeling method so that all of the viable tumor cells are distinguishable under a microscope from the non-tumor cells. In this embodiment, a tumor-specific fluorochrome-conjugated antibody can be used to specifically mark each of the tumor cells, yet not mark any of the hematopoietic cells. The labeled tumor cells are then microscopically identified within the population of non-tumor cells. A narrow, high power laser beam is thereafter focused on each of the identified tumor cells and a brief, lethal light pulse is delivered. The next tumor cell is then identified and killed, and so on until every marked tumor cell is destroyed.

In another embodiment, an antibody that selectively binds to hematopoietic cells, but not tumor cells, is used to identify hematopoietic stem cells. All of the tumor cells (eg: those that do not take up label) are identified and thereafter killed with a narrow, high power laser beam.

Yet another embodiment is a method of eliminating tumor cells from within a population of cells that includes non-tumor cells, including the steps of: a) labeling the population of cells so that the tumor cells can be distinguished from the non-tumor cells; b) locating one of the tumor cells by reference to the label; and c) killing the tumor cell by applying a pulse from a controlled energy source to the located tumor cell.

One additional embodiment is a method for enriching the number of stem cells in a hematopoietic cell population, including: a) labeling the hematopoietic cell population with a stem cell specific label; and b) applying a high-energy laser-light pulse to at least one of the un-labeled cells in the population.

Still another embodiment is a method for preparing isolated hematopoietic cells for re-introduction into a patient, including: a) labeling tumor cells within the isolated hematopoietic cells with a tumor cell specific label; and b) applying a high-energy laser-light pulse to at least one of the labeled cells in the population.

Another embodiment of the invention is an *in vitro* method for ensuring that a first population of cells has been eliminated from a second population of cells, comprising. The method includes: labeling the first population of cells so that the first population of cells can be distinguished from the second population of cells; placing the first population of cells and the second population of cells in a stationary position on a surface; locating one cell in the first population of cells by reference to the label; applying a first pulse from a controlled energy source to the one cell; determining if the one cell was killed by the first pulse; and applying a second pulse from a controlled energy source if the one cell was not killed.

A further embodiment of the invention is a method of eliminating tumor cells from within a cell population that includes non-tumor cells. The method includes the steps of: labeling the non-tumor cells so that the non-tumor cells can be distinguished from the tumor cells; locating one of the tumor cells by identifying a non-labeled cell in the cell population; and killing the tumor cell by applying a pulse from a controlled energy source to the located tumor cell while the tumor cell is in a substantially stationary position on a surface.

#### Brief Description of the Drawings

FIGURE 1 is a diagram of one embodiment of an automated system for specifically targeting labeled cells. The system includes a computer, a camera, a broad-band light source and a laser.

FIGURE 2 is a diagram of another embodiment of an automated system for specifically targeting and killing labeled cells.

#### Detailed Description

A targeted method is provided for removing contaminating tumor cells from a population of non-tumor cells. As discussed above, this method relates to medical procedures whereby populations of cells are removed and then re-introduced into patients as part of a therapeutic regime. Generally, the targeted method first employs a label that acts as a marker to microscopically identify and locate the contaminating tumor cells.

The chosen cellular marker can be any label that identifies and discriminates a contaminating tumor cell residing within a population of non-tumor cells. For example, anti-tumor antibodies that are conjugated to a fluorochrome can be used as specific labels (*See* Chapter 10 in A. Gee, Editor, Bone Marrow Processing and Purging, Part 5, CRC Press, Boca Raton, Florida, 1991). Numerous tumor-specific makers have been found on a wide variety of tumorigenic cells. For example, many surface markers that are specific to epithelial cells have been found on contaminating breast cancer cells in harvested hematopoietic cell populations. Such antibodies can be used to identify breast cancer cells within hematopoietic cell populations and therefore target them for the delivery of a lethal energy pulse. Similarly, other tumor-specific fluorochrome-conjugated antibodies can be used to identify other types of contaminating tumor cells.

It should also be noted that if no specific markers are available for the tumor cell population, the method can be implemented in an 'inverse' fashion. Specific markers for the non-tumor cell population can be used to identify those cells that are not tumorigenic. For example, in hematopoietic cell populations, the CD34 cell marker can be used to stain only the hematopoietic cells, but not the non-hematopoietic tumor cells. Any cell that does not have the marker is then killed by the delivery of a focused energy pulse. The remaining viable cell population will only contain the cells that possessed the cell marker (eg: CD34).

After the tumor cells are identified, a controlled energy source, such as a laser, collimated or focused non-laser light, RF energy, accelerated particle, focused ultrasonic energy, electron beam, or other radiation beam is used to deliver a targeted lethal energy pulse that individually kills each of the tumor cells. Because the tumor cells

can be specifically and uniquely identified within a population of non-tumor cells, the method can be used to completely eradicate just the contaminating tumor cells.

In one embodiment of the targeted method, the tumor cells are killed by simultaneous fluorescence and laser illumination. In a first step, the cell population is stained with a fluorescent stain that is specific for tumor cells. For example, antibodies that are specific for tumor cells and conjugated to fluorescent molecules could be used in this embodiment. The cell population is then illuminated by an appropriate light (e.g., ultraviolet light) so that the fluorescent tumor cells can be identified within the population of non-tumor cells.

The labeled cells are then placed in a Petri dish and placed within the system. If the system is in an automatic mode, a fluorescent light is illuminated and a CCD camera begins taking video pictures of the cells in the dish. A computer program running on an attached computer begins analyzing the pictures and identifying every one of the fluorescent cells. Each cell is targeted and destroyed with a laser beam. The computer confirms that each cell is killed by noting the change in fluorescence before and after the laser has been activated.

In another embodiment, contaminating cells in biopsies are removed from cancer patients. The *in vitro* establishment of primary human tumor cell lines from many tumor types is complicated by the presence of contaminating primary cell populations that have superior *in vitro* growth characteristics over tumor cells. For example, contaminating fibroblasts represent a major challenge in establishing many cancer cell lines. The disclosed system could be used to particularly label and destroy the contaminating cells, while leaving the biopsied tumor cells intact. Accordingly, the more aggressive primary cells will not overtake and destroy the cancer cell line.

In a related application, the present system could be used to remove contaminating cells in inocula for tissue engineering and cell therapy applications. Cell contamination problems exist in the establishment of primary cell cultures for tissue engineering applications and in the implementation of cellular therapies. In particular, chondrocyte therapies for cartilage defects are hampered by impurities in the cell populations derived from cartilage biopsies. Accordingly, the present system could be used to specifically remove these types of cells from the inocula. For example, a cartilage biopsy can be taken and the sample grown under conventional conditions. The growing culture is then stained with a specific label for the contaminating cells. The entire cell population is then placed within the system described below and the labeled, contaminating cells are destroyed.

In another embodiment, the significant risk and severity of Graft Versus Host Disease (GVHD) in the allogeneic Human Stem Cell (HSC) transplant setting can be combated. By using the system described herein, it is possible to define and control a patient's T cell content. This type of control is particularly difficult with existing technology, because over-depletion of T cells results in loss of the known beneficial graft versus leukemia effect that is observed in allogeneic transplants. Thus, some level of T cells is beneficial to the patient in order to attack, for example, leukemia cells. However, too high of a T-cell level leads to general immune system attacks on the recipient's tissues. The present system and methods are capable of precisely controlling the number of T cells in any allogeneic transplant.

One embodiment of an automated targeting system 10 is illustrated in Figure 1. A computer system 12 communicates through a cable 14 to a camera 16. The computer system can be any commercially available computer

that can receive and analyze video data. One example of such a computer system is an Intel Pentium Pro computer running Microsoft Windows 95 software.

Although a video camera is illustrated in the embodiment disclosed in Figure 1, the camera 16 can be any type of image gathering equipment known to those in the art. The camera 16 is mounted onto a support 20 so that it is held in a generally vertical orientation with respect to the ground.

The lens 22 of the camera 16 is designed to mate with a microscope 24, through an eyepiece 26. Thus any image that is transmitted into the microscope 24 is sent through the eyepiece 26 to the lens 22 of the camera 16.

The camera 16 transmits the images that it captures to the computer 12 through the cable 14. In this manner, the computer 12 captures and analyzes any images that are presented to the microscope. For example, the microscope 24 may be focused on cells in a dish 30. The images of the cells will be transmitted from the microscope 24 to the camera 16, and finally be sent to the computer 12. The computer 12 can then run software to analyze the images that are captured.

The computer 12 is also in communication through a second cable 32 to a laser 34. The laser is mounted to an electronically controlled swivel 36 so that its position relative to the support 20 can be altered by receiving signals from the computer 12. As shown, the laser 34 can be adjusted to aim a light beam through the microscope 24 optics while the target cell is substantially stationary. In addition, the laser 34 can be swiveled to send a light beam directly to the dish 30.

In the context of the present invention the term "dish" has a broad meaning. It is intended to encompass any surface capable of retaining a thin layer of fluid such as cellular medium. The dish may incorporate or be associated with cooling apparatus to keep the fluid in the dish below ambient temperature during operation of the methods described herein. It may also be desirable, but not essential, to blanket the surface of the fluid with an inert atmosphere such as nitrogen or argon.

Although the embodiment illustrated in Figure 1 includes an electronically controlled swivel 36 to aim the laser, other methods are anticipated. For example, an electronically controlled mirror could be placed between the laser and its target so that rotation of the mirror is used to aim the laser. Other mechanisms for aiming a laser, such as beam splitters, mirrors or prisms that are known in the art are meant to be included within the scope of the invention.

A broad-band light source 40 is also in communication with the microscope 24 through an adapter 42. The adapter 42 allows light emissions from the broad-band light source to illuminate cells that are placed on the dish 30. Broad-band light sources are well known in the art, and many different types could be placed within the automated targeting system 10 to illuminate labeled cells that are placed on the dish 30.

Below and supporting the dish 30 is a computer-controlled, movable tray 48. The computer 12 can send signals along a communication cable 49 that cause the tray 48 to change its position relative to the laser 34 and microscope 24. Thus, to specifically target cells, the computer 12 can send signals along the cable 49 to the tray 48 that cause the dish 30 to be rolled into a particular position. By calculating the correct position, the laser can

be aimed towards a particular cell by moving the dish 30 into a chosen position. The coordinates of the chosen position can be determined by the computer 12 from analyzing cellular images that are gathered from the camera 16.

5 Software that is running on the computer 12 captures the images that are transmitted through cable 14 from the camera 16. If a set of labeled cells are placed in the dish 30, then the camera 16 will record a picture of the labeled cells. As discussed above, the cells can be illuminated with the broad-band light source 40. By overlaying a two-dimensional grid on top of the picture gathered by the computer, the software can determine the X/Y coordinates of the cells that are labeled by looking for dark spots that are one cell width in diameter. The software then calculates the Cartesian coordinates of the labeled cells and specifically aims the laser 34 to those  
10 coordinates. By emitting a short, high-energy pulse, the laser can selectively kill each of the labeled cells and not damage any of the non-labeled cells.

The computer system 12 can also have sensors that detect fluorescent signals from stained tumor cells and then calculate the coordinate position of the first tumor cell to destroy. The computer system then aims a focused energy source, such as a laser or electronic beam, at the calculated position of the first tumor cell. The  
15 targeted tumor cell is thereby specifically destroyed within the non-tumor cell population. This is facilitated by the fact that the target cell is substantially stationary during the procedure.

In another exemplary system, the cells are mounted for movement on a computer-controlled X-Y table under a microscope coupled with a CCD digital camera. The laser is mounted to the microscope such that it is aimed at a specific fixed location within the field of view. The computer then scans continuously across a first row of cells  
20 in the X direction until a stained tumor cell is visualized.

The computer then moves the X-Y table until the tumor cell is in the target area of the laser; the laser is pulsed to destroy the cell; then the X-Y table is returned to its previous position and is moved along the X direction until a first row is scanned and all tumor cells within that row are destroyed.

The X-Y table is then moved in the Y direction until a second row is visualized, overlapping the first row  
25 by a few cells. The table is then moved in the -X direction, tumor cells are destroyed as above, and the procedure is repeated until the entire cell population has been scanned. Preferably, the computer software automatically identifies and targets the cells. However, it is also contemplated that an operator-controlled pointing device (e.g., trackball, joystick, or mouse) could be used to locate and mark tumor cells on a display screen and/or to control movement of the X-Y table. Of course, many variations of the computer control system are possible, including  
30 alternative methods for scanning the cells and movement of the laser relative to the microscope (e.g., in the Y direction only, or anywhere within the field). It is also envisaged that the dish/table could remain substantially stationary and the microscope/laser combination could move to target labeled cells.

For example, the microscope head and laser targeting system can be moved while the flask or Petri dish holding the cells is stationary on a surface. Additionally, the laser system can fire from either from or above the  
35 cell population. Because the laser targeting system is focussed through a microscope, the laser beam can be directed to different focal planes. Thus, cells that are located at different vertical heights can be specifically killed by

targeting the laser beam to varying focal planes. For example, once the computer, through a detector, has identified a cell at a particular X and Y coordinate, the laser can be fired into several focal planes at those coordinates. This ensures that the cell, no matter where it is located in the vertical plane, will be killed by the laser.

5 The entire population of tumor cells can be automatically destroyed by repeating this targeted procedure for every tumor cell in the population. The procedure of labeling, identifying and killing each tumor cell can also be carried out manually, whereby each labeled tumor cell is microscopically identified by an operator and the laser beam is then focused by the operator on the illuminated tumor cells. A combined laser/microscope system that allows manual targeting of cells is available from Photonic Instruments (Arlington Heights, IL).

10 For example, a Nikon7 Diaphot 300 microscope that allows visualization of successive adjacent fields can be utilized to view the entire cell population in a reasonable time. An exemplary cell population of  $1.5 \times 10^8$  cells, at confluence, will occupy an area of  $300 \text{ cm}^2$ . Assuming a field of view of  $2 \times 1.5 \text{ mm}$  at a magnification of 4X, the microscope would need to scan a  $100 \times 100$  matrix, or 10,000 fields in total. A manual operator can scan perhaps 500-1000 fields per hour, so the total manual procedure would take approximately 10-20 hours. The automated procedure would be much faster.

15 Once the entire population of cells has been treated with an identification agent, such as a fluorescent-labeled antibody, and then killed by an energy pulse, the cell population is washed according to the patient's individual clinical needs. The population of treated cells is thereafter ready to be transplanted into the chosen patient. As discussed above, the patient is normally the same person as the original donor of the cell population.

20 A preferred focused energy source for the targeted method is a laser that transmits wavelengths of light in the range of 375 to 900 nanometers. One such laser is made by Photonic Instruments (Arlington Heights, IL). As is known, lasers provide a monochromatic light source with highly specific directionality, making it possible to aim a microscopically focused beam of energy having a diameters of only 0.5 microns.

25 High precision, both in terms of spatial and temporal resolution, in the delivery of light energy can be achieved at predefined wavelengths. The most advantageous wavelength that will kill a particular tumor cell can be determined experimentally and employed for the most advantageous results. In addition, pulse lengths of approximately 10-100 nanoseconds can be used to specifically kill only a single cell in the population.

30 As an alternative, broad-band light sources (e.g. as obtained from a Xenon Lamp) can be narrowly focused by employing an automated iris to provide a controlled energy source. A high-powered broad-band light-source can be focused on areas as small as one square-micron and thus selectively used to kill a single tumor cell within a population of non-tumor cells.

35 The power of the light source, and the duration of the light pulse, can be adjusted to achieve the desired result of specifically killing a particular tumor cell. Laser pulse lengths can be as short as 2 to 6 nanoseconds, and light energy can be emitted at up to 50 microjoules or more. However, the total amount of light energy provided to the designated target cell is preferably selected not to cause substantial boiling of the targeted cellular material or the surrounding medium.

Locally heating the cellular medium to the point of boiling can cause the field of view to become clouded, thus disrupting an automated targeting system. In addition, there is a risk that non-tumor cells that are located next to the targeted cell will become damaged. In one embodiment, the total light power delivered will minimally disrupt the cellular membrane thereby leading to eventual cell death through the loss of cytoplasmic components. Alternatively, the total light power can be chosen to irreversibly damage cellular components, thus leading to cell death without destruction of the cellular membrane.

To microscopically observe the tumor cells, the total cell population can be advantageously placed in a nominally flat surface so that a large number of cells appear in a single focal plane. The density of cells on this surface can, in principle, be at any value. However, the cell density should be as high as possible to minimize the total surface area required for the procedure. If the contaminating cells are clearly identifiable, the total cell population can be placed on the surface at confluence (about 500,000 cells per square centimeter).

If the population of harvested cells are hematopoietic cells that were removed as part of a bone marrow transplant, then enriching the population for only the desired stem cells will allow the population to be observed in a smaller total area. For instance, to observe a CD34-enriched population of 350 million (5 million per kilogram in a 70 kilogram patient) hematopoietic cells, the total surface area required is approximately 700 square centimeters. However, if the hematopoietic stem cell enrichment procedures included screening for multiple stem cell specific markers, the total number of target cells would decrease, thereby requiring a smaller total area for the targeted procedure. As is known, hematopoietic populations can be enriched for stem cells based on expression of the stem cell markers Thy-1, CD38, CD15, CD11b, Gly-A, CD3, or CD19. In addition, the cells can be enriched by the well-known techniques of deep bed filtration or counterflow elutriation.

If the tumor cell detection is not hampered at cell confluency, then it may be desirable to keep the target cell population at subconfluency so that collateral damage to neighboring cells is minimized. However, because the total number of cells is large compared to the number of tumor cells, some collateral damage to surrounding cells can be tolerated. Detection and location of labeled tumor cells in a three dimensional space using confocal microscopy would remove the need for a flat surface and the identification and focusing of the lethal light source can take place though a volume in which the cells are placed.

After the tumor cells have been specifically destroyed, the cellular debris can be removed by washing the cells at a low temperature. Depending on the projected length of the procedure, the target cell population can be cooled to 4°C so that the contaminant removal process will proceed with only a minimum degradation in physiological performance of the non-tumor cell population. This can be especially important when the non-tumor cells are hematopoietic cells, since they are subject to degradation at room temperature. A thermoelectric cooling device can be used during this procedure to cool the cell population.

As is known, cooling the cell population to 4°C eliminates all cell motion. The surface on which the cells are placed can also be coated with polycationic compounds, such as poly-L-Lysine, so that the cells stick tightly.

The following example illustrates one embodiment of the targeted method for removing tumor cells from a population of hematopoietic cells.

Example 1Hematopoietic Stem Cell Transplantation

5 A patient with a metastatic tumor and in need of an autologous bone marrow transplant is identified by a physician. As a first step in the treatment, the patient undergoes a bone marrow harvesting procedure. In this procedure, the patient is placed under general anesthesia in an operating room. The patient's posterior iliac crest is then punctured multiple times by the surgeon and the bone marrow is aspirated.

10 The harvesting procedure results in retrieval of approximately  $1 \times 10^9$  hematopoietic cells. The harvested cells are enriched for hematopoietic cells by being first run over an immunoaffinity column that selects for cells having the CD34 hematopoietic cell-specific surface antigen. To enrich the harvested cell population even further for stem cells, a second selection is performed by running the harvested cells over an immunoaffinity column that is bound with the anti-stem cell antibody, Thy-1.

15 After conventional elution from the column, the enriched hematopoietic cell population is thereafter contacted with CD34 antibodies that have been conjugated to a fluorochrome. The labeled antibodies specifically bind to the hematopoietic cells, but not the tumor cells. The cell population is then placed on a nominally flat surface at confluence. The cell density is a monolayer of approximately 500,000 cells per square centimeter. An ultraviolet light that illuminates the fluorochrome-conjugated CD34 antibody is turned on to identify the hematopoietic cells.

An operator then targets a laser light from a Nitrogen laser. A laser beam with a wavelength of 375 nanometers and pulse length of five nanoseconds is used to manually target and kill each tumor cell that is identified because it did not fluoresce.

20 Once the non-fluorescent tumor cells have been destroyed, the remaining population is cryopreserved. Before the cells are re-introduced, the patient is given chemotherapeutic treatment to destroy the tumor cells that have metastasized throughout the patient's body. Following this treatment, the isolated cells are prepared for re-introduction by rapid thawing at 37°C. The tumor cell-free hematopoietic stem cells are then transplanted into the patient. The patient subsequently recovers with no remission of the original cancer.

25 Although the bone marrow harvesting and re-introduction procedures are usually carried out by a physician, the operator for the method of the present invention needs no medical training.

Example 2

30 An experiment was performed to determine if Propidium Iodide (PI) could be used to confirm that a target cell, in a population of cells, was killed by laser treatment. As is known in the art, Propidium iodide is specific to double-stranded nucleic acids. Since PI can diffuse into the nucleus of dead cell, but can not penetrate the membrane of viable cell, we tested whether viability of target cells could be confirmed by PI color development in their nuclei.

35 Additionally, PI has an absorbance peak at 493 nm, which is close to the absorbance peak of the label phycoerythrin (PE) at 480 nm. Thus, the PI and PE could be activated by the same wavelength of light. In addition,

PI has an emission peak of 617 nm, which was far enough to distinguish it from that of PE at 578 nm. Thus, a positive PE reading could be distinguished even when PE-labeled antibodies were bound to the cell

#### *Cell Line*

The human hematopoietic cell line KG1a was obtained from the American Type Culture Collection (Rockville, MD). Cells were received cryopreserved and then rapidly thawed and suspended in IMDM (Life Technologies, Grand Island, NY) containing 4mM L-glutamine, 3.024 g/L sodium bicarbonate and 20 % fetal bovine serum. The cell line was passed every 3 to 4 days and maintained in an incubator at 37 °C, 95 % humidity and 5 % CO<sub>2</sub> (as recommended by ATCC guidelines).

#### 10 *Cell Staining*

The KG1a cells were stained by direct immunofluorescence staining of cell surface antigens using phycoerythrin (PE)-conjugated monoclonal antibodies against the human CD34 membrane antigen, as recommended in the manufacturer's guidelines (Becton Dickinson Immunocytometry Systems, San Jose, CA). The labeled KG1a cells (targets) were mixed with unstained KG1a cells (control population) in a ratio of 1:100.

15 Propidium iodide (Sigma Chemical Company, St. Louis, MO) was added to make a final concentration of 5 µg/mL in a cell suspension of 1x10<sup>6</sup> cells/mL. Each 20 µL aliquot of a mixed cell suspension of 1.0x10<sup>6</sup> cells/mL was added into wells of 384 well plate (Nalge Nunc International, Naperville, IL), and after 30 minutes for cell settling, image capture and laser ablation were carried out.

#### 20 *Cell Image Acquisition*

Figure 2 illustrates a schematic diagram of one embodiment of a laser ablation system 75. The system includes a Charged Couple Device (CCD) camera 77 linked to a personal computer 80. The personal computer 80 was a SGI O<sub>2</sub> workstation (Silicon Graphics, Mountain View, CA). Digitized cell images were captured by the cooled CCD camera 77 (Photometrics Inc., Tucson, AZ) that had been installed in an inverted Diaphot 300 fluorescent microscope 25 (Nikon Inc., Melville, NY) with a 20x objective 84.

A selective fluorescent filter 86 (Set B2A; Chroma Technology Corp., Brattleboro, VT) was used to acquire a bright PE emission image. The captured images were displayed and processed by Isee™ software (Invision Corp., Durham, NC) running on the personal computer 80.

The software provided complete automation for the system including stage tool operation, z-direction focus, 30 coordination of regions of interest (e.g. targets) and laser control. There were 63 viewing image fields in each well of the 384 well plate and stepwise movement between fields was correctly controlled by the stage tool software routine within Isee. Each target cell 90 in the viewing field was identified under given parameters such as brightness, size and shape, and their locations were coordinated and marked by a feature extracting function in the Isee™ software. During the procedure, the target cells remained stationary on the surface 92 of their flasks.

### *Laser Ablation*

A certified class IIIb nitrogen laser 94, model VSL-337 (The Laser Science Inc., Newton, MA) equipped with a beam splitter 96 and a Coumarin 440 dye cell 98 unit (Photonic Instruments, Arlington Heights, IL) was used to kill individual cells. The beam splitter 96 passed light from the laser 94, but refracted the blue light emitted from a Mercury arc lamp 99. Thus, light from the mercury arc lamp 99 and laser 94 were sent along the same pathway towards the target cell 90.

The mercury arc lamp transmitted light having a wavelength range between 450 nm and 490 nm. However, as indicated in Figure 2, the blue light from the mercury arc lamp 99 was passed through a D470/40 excitation filter 100 (Chroma Technology Corp., Brattleboro, VT) in order to activate the PE conjugates attached to the CD34 surface antigen of the KG1a cells. Accordingly, the same pathway carried the excitation light and laser light.

The combination of laser light and excited mercury arc lamp light was then passed into a second beam splitter 102. The beam splitter 102 refracted the light from the laser 94 and mercury arc lamp 99 through the microscope objective 84 to the target cell 90. The beam splitter 102 also transmits the light that is illuminated by the labeled target cell through a filter 104 and into the CCD camera 77.

It should be noted that during a normal procedure, the laser light is only transmitted for brief instances. In contrast, the mercury arc lamp light is normally transmitted constantly so that each of the labeled cells will reflect light through the beam splitter 102, filter 104 and into the CCD camera 77.

A pulse generator 105 links the laser 94 to provide the shortest burst of laser light necessary to kill the target cell 90. Shorter pulse lengths are more desirable because there is less chance that surrounding cells will be damaged through local heating of the cellular media.

Digital images of a field of stained target cells were captured and their locations were coordinated and marked by the Isee software system. Because the arc lamp 99 produces a broad-band light that fluoresces every labeled cell in on the dish, the computer system was able to gather and process an entire field of view at one time.

As cells were being fired upon by short pulses of light from the laser, a vertically movable z-motor 110 provided a mechanism for specifically focusing the laser light onto differing focal planes in the dish. The z-motor 110 is an electronically controlled motor attached to the objective 84. The z-motor is connected so that the objective 84 can be moved up and down in the vertical direction in order to fine focus the laser into various focal planes. In addition, the z-motor can be controlled by the computer system 80 in order to focus the laser light pulse from the bottom of the target cell 90 to the top of the target cell. Focussing the laser pulse with the z-motor compensated for cell position variations in the vertical direction caused by imperfectly flat tissue culture plate surfaces.

### **Determination of Successful Cell Destruction**

After each cell was pulsed with the laser, the computer analyzed the spectrum at the PI emission peak of 617 nm. If the cell was killed by the laser, a peak emission at 617 nm would be found at the cell's original

location. If a PI emission peak was not found, the laser would send another pulse of light energy to the cell's location. In this manner, each tumor cell in the population was confirmed to have been killed.

#### Conclusion

5           This invention enables physicians to effectively treat patients in need of hematopoietic cell transplants without the risk of relapse due to re-introduction of cancerous cells. The targeted method described herein provides a method for the complete or almost complete removal of contaminating tumor cells from a hematopoietic stem cell or other cell population.

10           The invention may be embodied in other specific forms without departing from its spirit or essential characteristics. The described embodiments are to be considered in all respects only as illustrative and not restrictive. The scope of the invention is, therefore, indicated by the appended claims rather than by the foregoing descriptions. All changes which come within the meaning and range of equivalency of the claims are to be embraced within their scope.

I claim:

1. An *in vitro* method of eliminating tumor cells from within a population of cells that includes non-tumor cells, comprising the steps of:

5 a) labeling said population of cells so that said tumor cells can be distinguished from said non-tumor cells;

b) locating one of said tumor cells by reference to said label; and

c) killing said tumor cell by applying a pulse from a controlled energy source to said located tumor cell while said tumor cell is in a substantially stationary position on a surface.

10 2. The method of Claim 1, wherein steps b) and c) are repeated for every tumor cell in said population of non-tumor cells.

3. The method of Claim 1 or 2, wherein said population of non-tumor cells is a population of hematopoietic cells.

4. The method of any one of Claims 1 to 3, wherein said population of hematopoietic cells is comprised of a population of hematopoietic cells that has been enriched for hematopoietic stem cells.

15 5. The method of Claim 4, wherein said hematopoietic stem cell population is enriched based on expression of the CD34 surface protein.

6. The method of Claim 4, wherein said hematopoietic stem cell population is enriched based on expression of a cell marker selected from the group consisting of: Thy-1, CD38, CD15, CD11b, Gly-A, CD3, and CD19.

20 7. The method of Claim 4, wherein said enriched cell population is created by using deep bed filtration or counterflow elutriation.

8. The method of any one of Claims 1 to 7, wherein steps b) and c) are performed under the control of a computer.

9. The method of any one of Claims 1 to 7, wherein said controlled energy source is a laser.

25 10. The method of Claim 9, wherein the wavelength of light emitted from said laser is between 375 nanometers and 900 nanometers.

11. The method of any one of Claims 1-10, wherein said label is specific for said tumor cells.

12. The method of any one of Claims 1-11, wherein said label is specific for said non-tumor cells.

30 13. An *in vitro* method for enriching the number of stem cells in a hematopoietic cell population, comprising:

a) labeling said hematopoietic cell population with a stem cell specific label; and

b) applying a high-energy laser-light pulse to at least one of the un-labeled cells in said population while said stem cell is in a substantially stationary position on a surface.

35 14. The method of Claim 13, wherein said applying step comprises applying said high-energy laser-light pulse to substantially all of the un-labeled cells in said population.

15. The method of Claim 13 or 14, wherein said stem cell specific label is CD34.

16. The method of any of Claims 13 to 15 wherein the wavelength of light emitted from said laser is between 375 nanometers and 900 nanometers.

17. The method of any of Claims 13 to 16, wherein said applying step is performed under the control of a computer.

5 18. An *in vitro* method for preparing isolated hematopoietic cells for re-introduction into a patient, comprising:

a) labeling tumor cells within said isolated hematopoietic cells with a tumor cell specific label; and

10 b) applying a high-energy laser-light pulse to at least one of the labeled cells in said population while said labeled cell is in a substantially stationary position on a surface.

19. The method of Claim 18, wherein said applying step comprises applying said high-energy laser-light pulse to all of the labeled cells in said population.

20. The method of Claim 18 or 19 wherein the wavelength of light emitted from said laser is between 375 nanometers and 900 nanometers.

15 21. The method of any of Claims 18 to 20, wherein said applying step is carried out automatically with the assistance of a computer.

22. An *in vitro* method for eliminating a first population of cells from a second population of cells, comprising:

20 a) labeling said first population of cells so that said first population of cells can be distinguished from said second population of cells;

b) placing said first population of cells and said second population of cells in a stationary position on a surface;

c) locating one cell in said first population of cells by reference to said label;

d) applying a first pulse from a controlled energy source to said one cell;

25 e) determining if said one cell was killed by said first pulse; and

f) applying a second pulse from a controlled energy source if said one cell was not killed.

23. The method of Claim 22, wherein the method of labeling said first population of cells comprises labeling tumor cells.

30 24. The method of Claim 22 or 23, wherein the method of eliminating a first population of cells from a second population of cells comprises eliminating a first population of tumor cells from a second population of non-tumor cells.

25. The method of any of Claims 22 to 24, wherein the method of applying a first pulse comprises applying a laser pulse.

35 26. The method of any of Claims 22 to 25, wherein the method of applying a second pulse comprises applying a laser pulse.

27. A method of eliminating tumor cells from within a cell population that includes non-tumor cells, comprising the steps of:

a) labeling said non-tumor cells so that the non-tumor cells can be distinguished from said tumor cells;

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b) locating one of said tumor cells by identifying a non-labeled cell in said cell population; and

c) killing said tumor cell by applying a pulse from a controlled energy source to said located tumor cell while said tumor cell is in a substantially stationary position on a surface.

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28. The method of Claim 27, wherein the step of killing said tumor cell comprises applying a laser pulse.

29. The method of Claim 27 or 28, wherein the step of killing said tumor cell comprises applying a controlled energy source to said located tumor cell while said tumor cell is in a stationary position on a nominally flat surface.

15

30. The method of any of Claims 27 to 29, wherein the step of killing said tumor cell comprises applying a controlled energy source to a located tumor cell while said cell population is at confluence.

**AMENDED CLAIMS**

[received by the International Bureau on 4 September 1998 (04.09.98);  
original claims 1-3,8,13 and 18 amended;  
remaining claims unchanged (3 pages)]

- 5 1. An *in vitro* method of eliminating tumor cells from within a population of cells that includes non-tumor cells, comprising the steps of:
- a) labeling said population of cells so that said tumor cells can be distinguished from said non-tumor cells;
  - b) illuminating the entire population of cells;
  - c) capturing an image of the illuminated population of cells;
  - 10 d) determining at least the two dimensional coordinates of a tumor cell in the population of cells by reference to the captured image and said label; and
  - e) killing said tumor cell by applying a pulse from a controlled energy source to the coordinates of said tumor cell while said tumor cell is in a substantially stationary position on a surface.
- 15 2. The method of Claim 1, wherein steps d) and e) are repeated for every tumor cell in said population of non-tumor cells.
3. The method of Claim 1 or 2, wherein said non-tumor cells are a population of hematopoietic cells.
4. The method of any one of Claims 1 to 3, wherein said population of hematopoietic cells is comprised of a population of hematopoietic cells that has been enriched for hematopoietic stem cells.
- 20 5. The method of Claim 4, wherein said hematopoietic stem cell population is enriched based on expression of the CD34 surface protein.
6. The method of Claim 4, wherein said hematopoietic stem cell population is enriched based on expression of a cell marker selected from the group consisting of: Thy-1, CD38, CD15, CD11b, Gly-A, CD3, and CD19.
7. The method of Claim 4, wherein said enriched cell population is created by using deep bed filtration or counterflow elutriation.
- 25 8. The method of any one of Claims 1 to 7, wherein steps d) and e) are performed under the control of a computer.
9. The method of any one of Claims 1 to 7, wherein said controlled energy source is a laser.
10. The method of Claim 9, wherein the wavelength of light emitted from said laser is between 375 nanometers and 900 nanometers.
- 30 11. The method of any one of Claims 1-10, wherein said label is specific for said tumor cells.
12. The method of any one of Claims 1-11, wherein said label is specific for said non-tumor cells.
13. An *in vitro* method for enriching the number of stem cells in a hematopoietic cell population, comprising:
- a) labeling said hematopoietic cell population with a stem cell specific label;
  - b) illuminating the entire hematopoietic cell population;
  - 35 c) capturing an image of the illuminated hematopoietic cell population;
  - d) calculating at least the two-dimensional coordinates of any unlabeled cells in the hematopoietic cell population by determining their position on the image; and

e) applying a high-energy laser-light pulse to the coordinates of at least one of the unlabeled cells in said population while said stem cell is in a substantially stationary position on a surface.

5 14. The method of Claim 13, wherein said applying step comprises applying said high-energy laser-light pulse to substantially all of the un-labeled cells in said population.

15. The method of Claim 13 or 14, wherein said stem cell specific label is CD34.

16. The method of any of Claims 13 to 15 wherein the wavelength of light emitted from said laser is between 375 nanometers and 900 nanometers.

10 17. The method of any of Claims 13 to 16, wherein said applying step is performed under the control of a computer.

18. An *in vitro* method for preparing isolated hematopoietic cells for re-introduction into a patient, comprising:

15 a) labeling tumor cells within said isolated hematopoietic cells with a tumor cell specific label;  
b) illuminating the isolated hematopoietic cells;  
c) capturing an image of the isolated hematopoietic cells;  
d) determining at least the two dimensional coordinates of said tumor cells within the isolated hematopoietic cells by reference to said image and said label; and

20 e) applying a high-energy laser-light pulse to at least one of the labeled tumor cells at the determined coordinates while said tumor cells are in a substantially stationary position on a surface.

19. The method of Claim 18, wherein said applying step comprises applying said high-energy laser-light pulse to all of the labeled cells in said population.

20. The method of Claim 18 or 19 wherein the wavelength of light emitted from said laser is between 375 nanometers and 900 nanometers.

25 21. The method of any of Claims 18 to 20, wherein said applying step is carried out automatically with the assistance of a computer.

22. An *in vitro* method for eliminating a first population of cells from a second population of cells, comprising:

30 a) labeling said first population of cells so that said first population of cells can be distinguished from said second population of cells;

b) placing said first population of cells and said second population of cells is in a stationary position on a surface;

c) locating one cell in said first population of cells by reference to said label;

d) applying a first pulse from a controlled energy source to said one cell;

35 e) determining if said one cell was killed by said first pulse; and

f) applying a second pulse from a controlled energy source if said one cell was not killed.

23. The method of Claim 22, wherein the method of labeling said first population of cells comprises labeling tumor cells.

5 24. The method of Claim 22 or 23, wherein the method of eliminating a first population of cells from a second population of cells comprises eliminating a first population of tumor cells from a second population of non-tumor cells.

25. The method of any of Claims 22 to 24, wherein the method of applying a first pulse comprises applying a laser pulse.

10 26. The method of any of Claims 22 to 25, wherein the method of applying a second pulse comprises applying a laser pulse.

27. A method of eliminating tumor cells from within a cell population that includes non-tumor cells, comprising the steps of:

15 a) labeling said non-tumor cells so that the non-tumor cells can be distinguished from said tumor cells;

b) locating one of said tumor cells by identifying a non-labeled cell in said cell population; and

c) killing said tumor cell by applying a pulse from a controlled energy source to said located tumor cell while said tumor cell is in a substantially stationary position on a surface.

28. The method of Claim 27, wherein the step of killing said tumor cell comprises applying a laser pulse.

20 29. The method of Claim 27 or 28, wherein the step of killing said tumor cell comprises applying a controlled energy source to said located tumor cell while said tumor cell is in a stationary position on a nominally flat surface.

30. The method of any of Claims 27 to 29, wherein the step of killing said tumor cell comprises applying a controlled energy source to a located tumor cell while said cell population is at confluence.

25

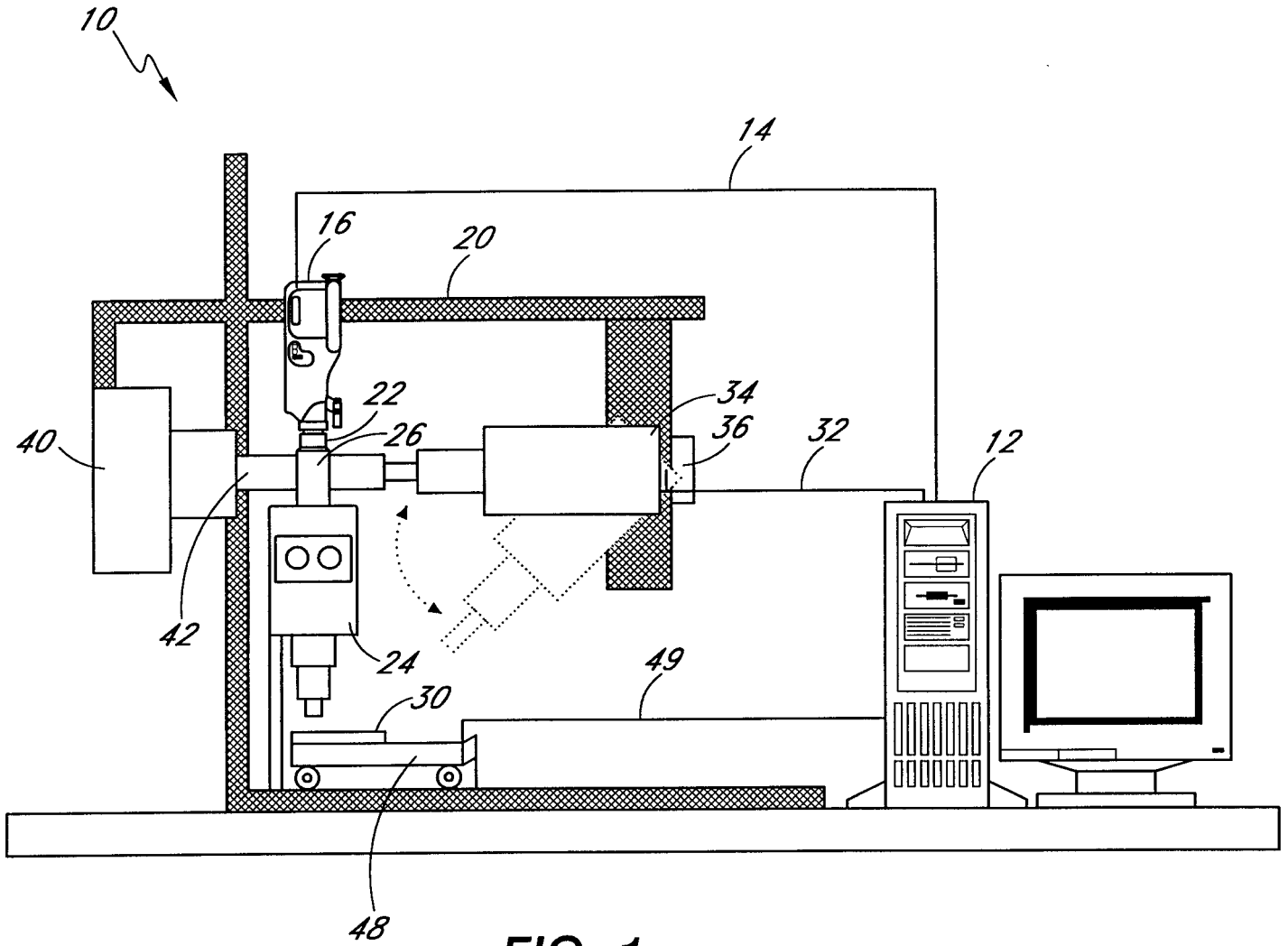


FIG. 1

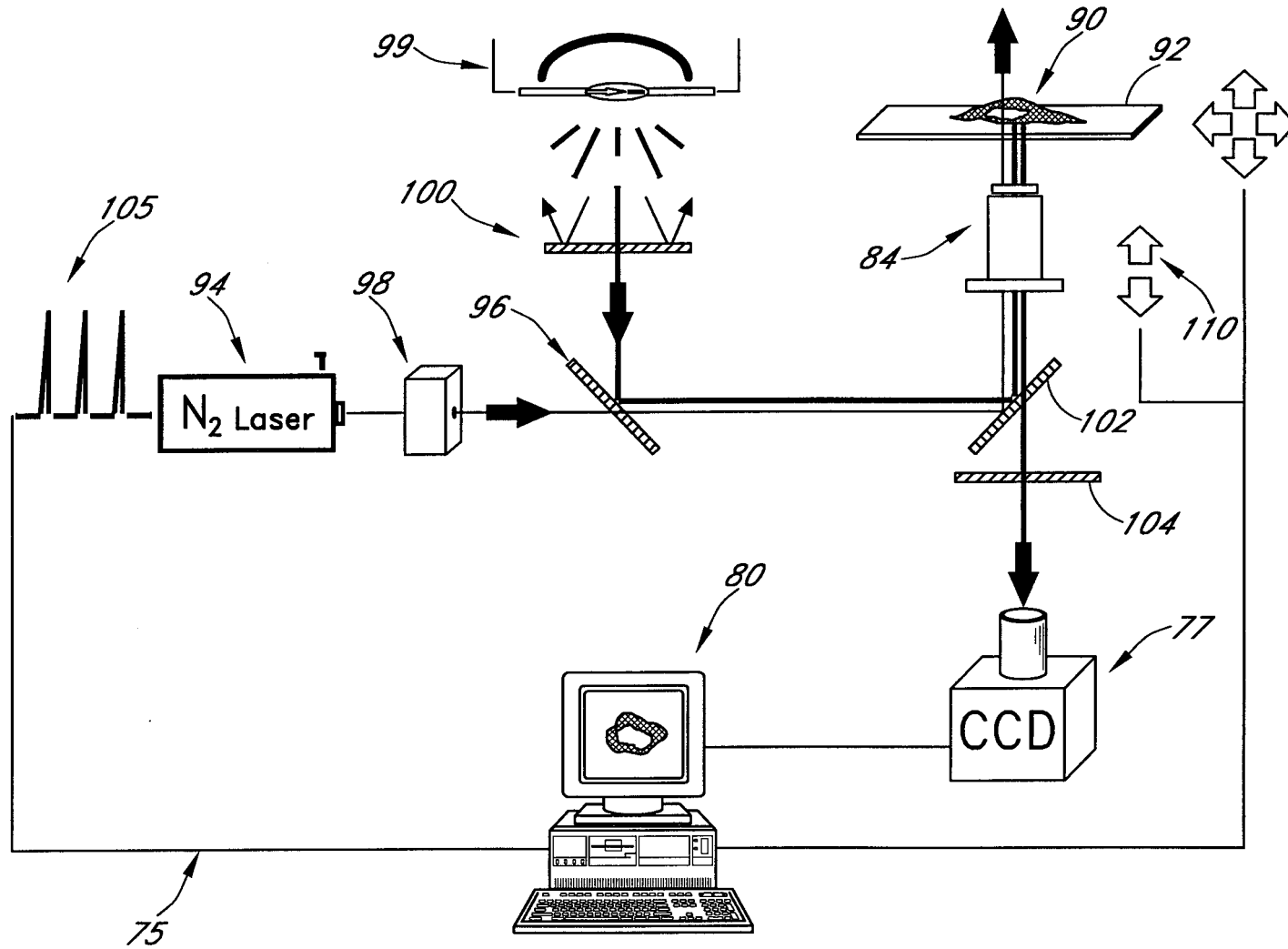


FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/06125

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : A61K 35/26; C12N 1/00, 13/00; C12Q 1/02  
US CL : 435/29, 173.1, 173.4, 173.7, 243; 424/577

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/29, 173.1, 173.4, 173.7, 243; 424/577

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS  
search terms: cancer?, tumor?, kill?, destroy?, select?, energy, laser

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,272,081 A (WEINREB et al.) 21 December 1993, see entire document especially columns 25-26.	1-2, 22-24, 27-29 ----- 3, 13-15, 18-20
X --- Y	US 4,624,915 A (SCHINDLER et al.) 25 November 1986, see entire document especially column 11.	1-2, 22-24, 27-29 ----- 3, 13-15, 18-20
X --- Y	US 5,035,693 A (KRATZER et al.) 30 July 1991, see entire document, especially columns 1 and 3.	1-2, 22-24, 27-29 ----- 3, 13-15, 18-20

Further documents are listed in the continuation of Box C.  See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* & * document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

13 MAY 1998

Date of mailing of the international search report

07 JUL 1998

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

JON P. WEBER, PH. D.

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/06125

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,188,633 A (KRATZER et al.) 23 February 1993, see entire document especially columns 1 and 3.	1-2, 22-24, 27-29 ----- 3, 13-15, 18-20
Y	US 4,395,397 A (SHAPIRO) 26 July 1983, see entire document.	1-3, 13-15, 18-20, 22-24, 27-29
Y	GAZITT et al. Purified CD34+ Lin- Thy+ Stem Cells Do Not Contain Clonal Myeloma Cells. Blood. 01 July 1995, Vol. 86, No. 1, pages 381-389, see entire document.	1-3, 13-15, 18-20, 22-24, 27-29
A	US 5,158,889 A (HIRAKO et al.) 27 October 1992.	1-3, 13-15, 18-20, 22-24, 27-29

INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/06125

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3.  Claims Nos.: 4-12, 16-17, 21, 25-26 and 30  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.